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TITLE: Novel Interventions for Heat/Exercise Induced Sudden Death and Fatigue

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**Introduction:** Exertional and/or environmental heat stroke (ES) and exertional rhabdomyolysis (ER) has been reported in patients with diagnosis of Malignant Hyperthermia (MH). MH is a life-threatening pharmacogenetic disorder caused by mutations in the ryanodine receptor type 1 gene (RYR1) encoding skeletal muscle calcium release channel. Our goal is to identify RYR1 mutations associated with enhanced susceptibility to EHS/ER/MH by enrolling subjects diagnosed with these conditions and performing genetic screening. We also proposed to evaluate the ability of AICAR to prevent the MH response in MHS mice and pigs.

<b>Task 1: Screen for RyR1 mutations</b>					
<b>1a: Sequencing</b>	<b>USUHS</b>	<b>45 human samples</b>	<b>XXXX</b>	<b>XXXX</b>	<b>XXXX</b>
<b>Milestone 1: Show that RyR1 mutations underlie cases of enhanced susceptibility to heat stroke</b>			<b>__XX</b>	<b>XX__</b>	
<b>Milestone 2 Publish findings</b>					<b>__XX</b>

**Body:** During the project period, 37 patients (out of 45 proposed) with a history of EHS/ER or MH were enrolled in this study. The RYR1 gene was screened in these individuals. RYR1 mutations and variants were identified in 13 of the 37 enrolled patients (see Table 1 attached). Four well known disease causative MH mutations (Arg163Cys, Gly2434Arg, Arg2454Cys and Arg2163His), two previously published MH-associated mutations and two novel variants were identified in the RYR1 gene. Of the four common mutations, the Arg2454Cys was identified in an African American patient with a positive CHCT (validated diagnostic test for MH susceptibility) and a history of ER. Since the Arg2454Cys mutation is characterized as causative for MH, identification of this mutation in a subject with ER further strengthens a link between MH and ER. A new variant, Gly4820Arg, was found in a family with a history of a death due to an awake MH-like event.

To date, 37 patients diagnosed with MHS, ER and/or EHS and their family members have been enrolled (Table 1). Of these, 27 are index cases and 10 are first degree relatives of two of the index cases, BU-05 and BU-12 (Fig. 1). Enrollment of family members is important for studying the genotype and phenotype relationships, and to understand the pathogenic significance of familial variants.

RYR1 gene mutations and variants have been found in 8 index cases, which account for 30% of the index cases. One patient, who died of an awake MH-like episode but was known to be MH susceptible, had a Phe41Ser novel RYR1 variant and a Gly2434Arg known MH-causative mutation. Of these two variants, the Gly2434Arg is one of the most common pathogenic mutations in the RYR1 gene. The presence of the second variant may have contributed to the fatal outcome in this case; however the functional significance of the second variant requires further studies. Another novel variant, Gly4820Arg, was found in the father of a child who had the same variant and died of an awake MH-like episode. This father was subsequently diagnosed MH susceptible by positive CHCT, and was further diagnosed with central core disease based on his muscle histopathology. Interestingly, another mutation at the same position, Gly4820Trp, was reported in association with MH susceptibility (Robinson et al., 2006).

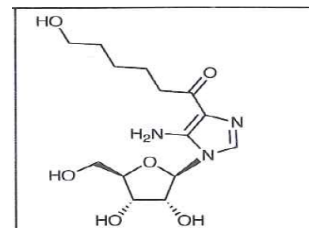
Three patients who presented with ER, and who were diagnosed MH susceptible by positive CHCT, did not have any RYR1 variants after complete screening of the gene. These patients will be further analyzed for CACNA1S and CASQ1 gene mutations. The RYR1 gene screening continues in 16 other patients.

<b>Task 2. Determine if AICAR's ability to rescue MH mice is due to effects on RyR1 or AMPK (animal protocol approved)</b>					
<b>2a. Indirect calorimetry to assess acute effects of AICAR, A769662, and derivatives for effects on VO<sub>2</sub> in each mouse. Mice: WT, MH, AMPK<math>\alpha</math>DN, AMPK<math>\gamma</math>CA, MH/AMPK<math>\alpha</math>DN, and MH/AMPK<math>\gamma</math>CA</b>	BCM	8-10 of each line (6) per year: 60 (total for 3 years is 180)	XXXX	XXXX	XXXX
<b>2b. Determine effects of AICAR on AMPK and RyR1 phosphorylation</b>	BCM	Same mice as in 2a, 2c, 3a, 3b	XXXX	XXXX	XXXX
<b>2c. Bilayer, Binding, Calcium Imaging</b> 6-8 week old mice are sacrificed and studies are performed with AICAR or A769662 added in vitro.	BCM	8-10 of each line/yr 60/year total for 3 years is 180	XXXX	XXXX	XXXX
<b>Milestone 3: Demonstration of AICAR's ability to regulate RyR1 activity</b>	BCM		__XX	XXXX	XXXX
<b>Milestone 4: Publication of these data</b>			__XX	__XX	__XX

Task 2 has been completed and the findings (milestone 4) published in 2012.

Lanner, J., Georgiou, D.K., Dagnino-Acosta, A., Ainbinder, A., Cheng, Q., Joshi, A., Chen, Z., Yarotsky, V., Oakes, J., Lee, C.S., Monroe, T., Santillan, A., Dong, K., Goodyear, L., Ismailov, I., Rodney, G.G., Dirksen, R., and Hamilton, S. AICAR Prevents Heat Induced Malignant Hyperthermia in RyR1 Mutant Mice Independent of AMPK Activation, Nature Medicine, 18: 244 – 251, 2012 PMID: 22231556 [PubMed - as supplied by publisher.

However we found that AICAR requires extremely high concentrations and hence we established a collaboration with Prof. Gennaro Piccilli to synthesize and test AICAR derivatives to find a more potent analog. We have a very good candidate (structure shown to the right) that is much more potent in [<sup>3</sup>H]ryanodine binding assays than AICAR. We are preparing to test it in the MH mice and then in the MHS pigs which, as discussed below, are not rescued by AICAR, most likely due to our inability to achieve an effective concentration.



<b>Task 3. Assess effects of chronic treatment with AICAR on MH response and endurance</b>					
<b>3a. Assess effects of chronic treatment with AICAR and A769662 on endurance, force and fatigue. Mice are treated for 6 weeks while on monitored running wheels. At the end of 6 weeks the mice are sacrificed and force-frequency and fatigue studies are performed.</b>	BCM	8-10 of each line/yr 60/year total for 3 years is 180	XXXX	XXXX	XXXX
<b>3b. Assess effect of chronic treatment with AICAR and/or A769662 on heat response by indirect calorimetry (mice are treated for 6</b>	BCM	8-10 of each line/yr 60/year total	XXXX	XXXX	XXXX

weeks while on monitored exercise wheels and then subject to indirect calorimetry)		for 3 years is 180			
Milestone 5: Determination of whether AICAR affect on endurance is due to effects of RyR1, AMPK or both.	BCM		XXXX	XXXX	XXXX
Milestone 6 Publication of Milestone 5					XX

These studies have been initiated but have not yet been completed. The mice will be treated with AICAR (600mg/kg) once a week for 6 weeks and we will assess the ER response, endurance and muscle performance

Task 4. Assess the ability of AICAR to prevent MH response in MHS susceptible pigs					
4a. Modify animal protocol	USUHS		X		
4b. Test AICAR in MHS pigs and controls	USUHS	6 normal and 6 MHS pigs	XX	XX	
4c: Therapeutic use of AICAR vs Dantrolene	USUHS	16 MHS pigs		-XX	XX
Milestone 7: Establish ability of AICAR to rescue MHS pigs				XX	XX
Milestone 8: Publish findings					XX

We found that AICAR, when administered as a pretreatment or as a rescue drug, was not effective as a treatment for heat-induced MH in the MH susceptible swine. This finding appeared to be in conflict with data obtained in the MH susceptible transgenic YS mouse model. We hypothesized that in the pig model AICAR (600mg/kg IV) may not achieve an effective concentration in the swine skeletal muscle. To test this possibility, we examined the effect of AICAR on isolated muscle preparations from the MHS pigs.

Six MHS swine were anesthetized as previously described with a total intravenous propofol infusion and euthanized at the end of the already approved in vivo protocol. While receiving propofol general anesthesia during the approved portion of the protocol, a 2 inch incision was made on the ventral surface of the swine's lower limb. The skin was shaved, cleaned and prepped with alcohol before incision. Employing sterile technique, a number 15 scalpel blade was used to incise the skin, while surgical scissors and forceps were used to expose the underlying muscle. The muscle was dissected and excised using scalpel, surgical scissors and forceps, and placed on tension in two specialized clamps. The muscle specimen was measured 1.0 inch by 0.5 inch, and it was immediately placed in a Krebs-Ringer solution. The muscle was taken to another laboratory for analysis in the *in vitro* contracture test (Table 2), using the standard procedures for the performance of the caffeine halothane contracture test as described by the North American Malignant Hyperthermia Group (Larach 1989). The results are shown in Table 2.

AICAR at 1mM and 10mM did not alter basal tension of muscle strips, ( $P = 0.96$  and  $0.86$ ) when compared to baseline tension of untreated control strips. Similarly AICAR (1 and 10mM) did not significantly alter halothane- or caffeine-induced contractures.

The results (Table 2) from the in vitro studies from MHS swine indicate that AICAR had no effect at both low and high concentrations on muscle tension development in basal conditions, nor did AICAR influence responses induced by caffeine and halothane challenges. This is consistent

with the previous report that AICAR is ineffective when administered intravenously in the MHS pig model.

#### **Key accomplishments:**

- Further support that RyR1 mutations underlie ER/EHS
- Publication of AICAR mechanism of action to prevent EHS
- Identification of AICAR derivative with higher potency
- Case report publication of awake MH

#### **Reportable Outcomes:**

- 37 patients diagnosed with MHS, ER and/or EHS and their family members have been enrolled (Table 1). Of these, 27 are index cases and 10 are first degree relatives of two of the index cases.
- RYR1 gene mutations and variants have been found in 8 index cases which account for 30% of the index cases.
- One patient who died of an awake MH-like episode had two RYR1 gene variants, Phe41Ser and Gly2434Arg. Of these two variants, the Gly2434Arg is one of the most common pathogenic mutations in the RYR1 gene.
- Two novel variants, Gly4820Trp and Phe41Ser were found in two independent cases.
- Published: Lanner, J., Georgiou, D.K., Dagnino-Acosta, A., Ainbinder, A., Cheng, Q., Joshi, A., Chen, Z., Yarotsky, V., Oakes, J., Lee, C.S., Monroe, T., Santillan, A., Dong, K., Goodyear, L., Ismailov, I., Rodney, G.G., Dirksen, R., and Hamilton, S. AICAR Prevents Heat Induced Malignant Hyperthermia in RyR1 Mutant Mice Independent of AMPK Activation, Nature Medicine, 18: 244 – 251, 2012 PMID: 22231 556 [PubMed - as supplied by publisher.
- We discovered an AICAR derivative that is more potent than AICAR
- AICAR at 1mM and 10mM did not alter basal tension of pig muscle strips, ( $P = 0.96$  and  $0.86$ ) when compared to baseline tension of untreated control strips.
- Similarly, AICAR (1 and 10mM) did not significantly alter halothane- or caffeine-induced contractures.
- AICAR is ineffective when administered intravenously in the MHS pig model, but the more potent derivative will now be tested
- Manuscript in print in Anesthesia and Analgesia. "Death in the Emergency Department: An unrecognized awake MH-like reaction in a six year old".

#### **Conclusions:**

- RYR1 mutations underlie a significant number of cases of exercise induced rhabdomyolysis and environmental heat stroke.
- AICAR prevents EHS in mice heterozygous for RYR1 mutation associated with MH in humans but not in pigs that are homozygous for a different mutation.
- Derivatives of AICAR may be more potent for preventing EHS/ER than AICAR

#### **References:**

Robinson R, Carpenter D, Shaw M-A, Halsall J, Hopkins P (2006) Mutations in *RYR1* in malignant hyperthermia and central core disease. *Hum Mutat* 27:977-89.

## Appendices:

**Table. 1 Results of RYR1 gene screening in enrolled subjects.**

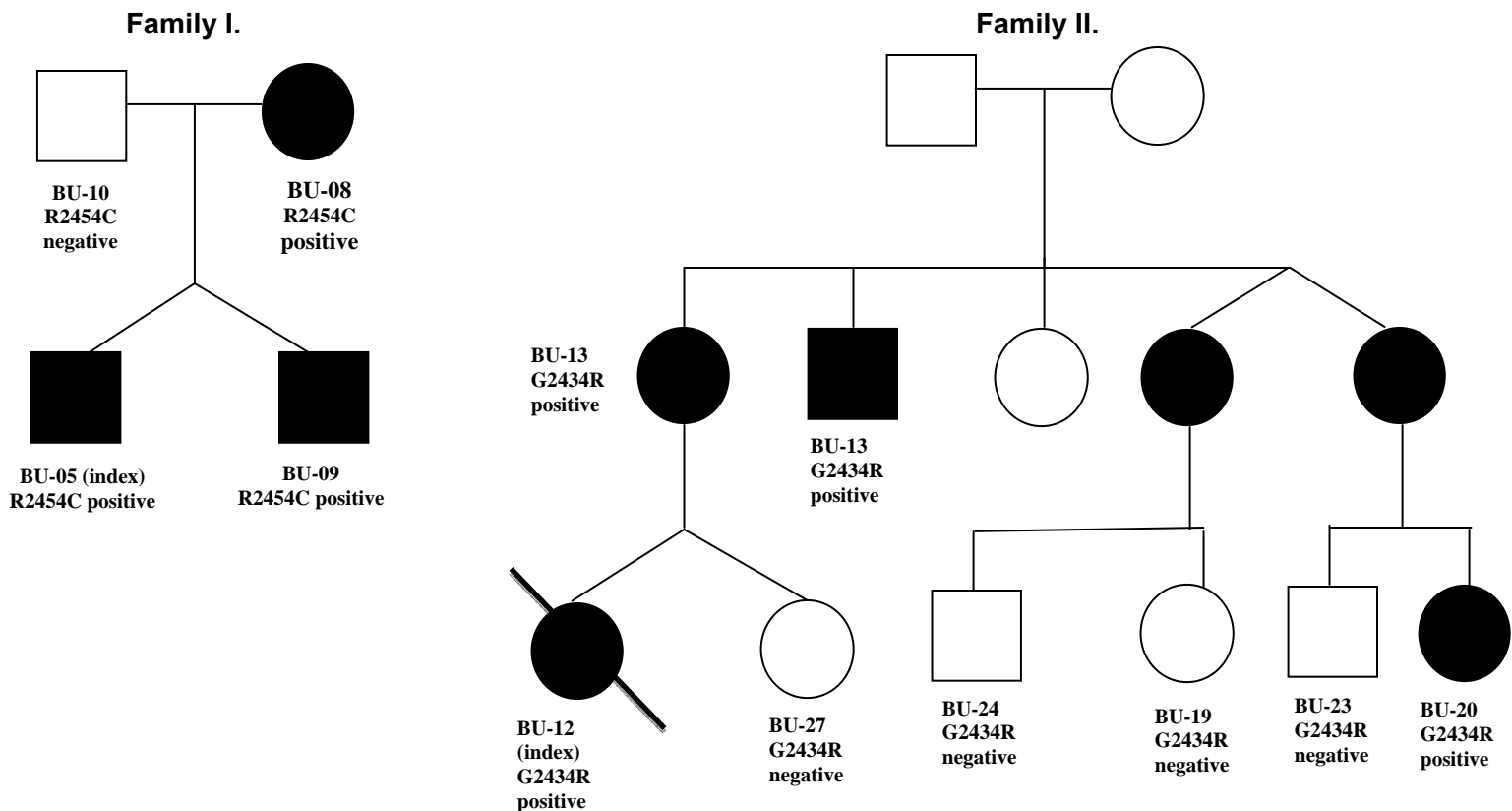
Report ID	Clinical history	CHCT Results	Index or Family members	Results of genetic screening
BU-01	Exercise induced rhabdomyolysis, heat related death in a family member	positive	index	<b>Gly4820Arg</b>
BU-02	Muscle pain with exercise post MH event	positive	index	<b>Arg163Cys</b>
BU-03	Exercise induced rhabdomyolysis, MHS	positive	index	In progress
BU-04	Exercise and heat intolerance, muscle cramping and MH	positive	index	<b>Asp3986Glu</b>
BU-05	Repeated Exercise induced rhabdomyolysis	positive	index	<b>Arg2454Cys</b>
BU-06	Repeated Exercise induced rhabdomyolysis	n/a	index	<b>Gly2434Arg</b>
BU-07	Death due to MH like event	n/a	index	In progress
BU-08	No known clinical history	positive	Mother of BU-05	<b>Arg2454Cys</b>
BU-09	Cardiac arrest during surgery	n/a	Twin brother of BU-05	<b>Arg2454Cys</b>
BU-10	No known clinical history	n/a	Father of BU-05	Negative for <b>Arg2454Cys</b> familial mutation
BU-11	Traumatic MH Episode	n/a	index	<b>Arg2163His</b>
BU-12	Death due to MH and Exercise/heat intolerance	n/a	index	<b>Phe41Ser &amp; Gly2434Arg</b>
BU-13	MH and heat related death in family member	n/a	Mother of BU-12	<b>Gly2434Arg</b>
BU-14	Exercise and heat intolerance	Positive	index	In progress



BU-15	Exertional Rhabdomyolysis	Positive	index	<b>Val4842Met</b>
BU-16	Exercise and heat intolerance	Negative	Index	In progress
BU-17	Family history of MH	n/a	Index	In progress
BU-18	Exercise/heat related intolerance, heat stroke and mother had suspected MH episode	n/a	index	In progress
BU-19	MH and heat related death in family member	n/a	1 <sup>st</sup> Cousin of BU-12	Negative for <b>Gly2434Arg</b> familial mutation
BU-20	MH and heat related death in family member	n/a	1 <sup>st</sup> Cousin of BU-12	<b>Gly2434Arg</b>
BU-21	Exertional Rhabdomyolysis	Positive	index	In progress
BU-22	MH and heat related death in family member	N/A	Uncle of BU-12	<b>Gly2434Arg</b>
BU-23	MH and heat related death in family member	N/A	1 <sup>st</sup> Cousin of BU-12	Negative for <b>Gly2434Arg</b> familial mutation
BU-24	MH and heat related death in family member	N/A	1 <sup>st</sup> Cousin of BU-12	Negative for <b>Gly2434Arg</b> familial mutation
BU-25	Heat and Exercise intolerance	N/A	index	In progress
BU-26	Exertional Rhabdomyolysis/ Heat & Exercise Intolerance	Negative	index	In progress
BU-27	MH and heat related death in family member	N/A	Twin sister to BU-12	Negative for <b>Gly2434Arg</b> familial mutation
BU-28	Exertional Rhabdomyolysis	Positive	index	Genetic screen completed, negative for RYR1 mutation
BU-29	Exertional Rhabdomyolysis	Positive	index	In progress
BU-30	Exertional Rhabdomyolysis	Positive	index	Genetic screen completed, negative for RYR1 mutation
BU-31	Exertional Rhabdomyolysis	Positive	index	Genetic screen completed, negative for RYR1 mutation
BU-32	Exertional Rhabdomyolysis	Negative	index	In progress
BU-33	Exertional Rhabdomyolysis	Negative	index	In progress

BU-34	Exertional Rhabdomyolysis	Negative	index	In progress
BU-35	Exertional Rhabdomyolysis	Negative	index	In progress
BU-36	Exertional Rhabdomyolysis	Negative	index	In progress
BU-37	Exertional Rhabdomyolysis	Negative	index	In progress

**Pedigree Analysis (Fig.1)**



Pedigrees of two unrelated MHS and Exertional Rhabdomyolysis families. Filled symbols denote individuals with familial mutations and or carriers of familial mutations. Filled symbol with bisecting line indicates individual that died from MH/Exercise and heat intolerance (BU-12). R or C denote Arginine or Cysteine at amino acid 2454 sequence position; G or R denote Glycine or Arginine at amino acid 2434 sequence position of the RYR1 gene respectively. Unlabeled symbols denote individuals that were not screened under this protocol.

**Table 2. Effect of AICAR on basal tension and 3% halothane-, 2mM caffeine-induced contractures on muscle strips from MHS swine.**

<b>Treatment</b>	<b>Twitches (g)</b>	<b>Baseline (0 min) Tension (g)</b>	<b>Tension 30 min Post</b>	<b>Tension 90 min Post</b>	<b>3% Halothane-Induced Contracture (g)</b>	<b>2 mM Caffeine-Induced Contracture (g)</b>
Control	3.25 ± 0.41 n = 36	2.18 ± 0.08 n = 36	1.81 ± 0.11 n = 36	1.74 ± 0.13 n = 18	3.26 ± 0.36 n = 18	1.16 ± 0.13 n = 14
1mM AICAR	3.55 ± 0.68 n = 18	2.11 ± 0.11 n = 18	1.68 ± 0.09 n = 18	1.72 ± 0.14 n = 9	2.99 ± 0.46 n = 9	1.37 ± 0.28 n = 7
10mM AICAR	4.6 ± 0.95 n = 18	1.82 ± 0.12 n = 18	1.30 ± 0.13 n = 18	1.33 ± 0.15 n = 9	2.93 ± 0.32 n = 9	1.43 ± 0.18 n = 7

Data were analyzed using two way ANOVA and are presented as Mean ± SEM, n = number of muscle strips tested. Muscle strips from MHS swine were mounted in an organ bath and after initial equilibration period (10-15 min) muscle strips were treated with distilled water with and without (control) AICAR (1 and 10mM). Basal tension of strips was recorded continuously up to 90mins. After 90mins period, muscle strips were treated with either 3% halothane or 2mM caffeine.

## QUARTERLY REPORT FORMAT

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4. PI: Susan Hamilton & Sheila Muldoon/John Capacchione
5. Telephone No. 713-798-5704
6. Institution: Baylor College of Medicine & Uniformed Services University of the Health Sciences (USUHS)
7. Project Title: Novel Interventions for Heat/Exercise Induced Sudden Death and Fatigue
8. Current staff, with percent effort of each on project.

### **Baylor**

Susan Hamilton, Ph.D. 10 %                      Keke Dong 20 %  
Dimitra Georgiou, Ph.D. 100 %

### **USUHS**

Sheila Muldoon, M.D 5%                      Nyamkhishig Sambuughin, Ph.D. 5%  
John Capacchione, M.D 5%                      Rolf Bunger, M.D. 5%  
Francis O'Connor, M.D. 5%                      Tarina Wallace, MS 50%

9. Award expenditures to date (as applicable):

<b>This Qtr/Cumulative</b>	<b>This Qtr/Cumulative</b>
Personnel _____	Travel _____
Fringe Benefits _____	Equipment _____/_____
Supplies _____	Other _____
	<b>This Qtr/Cumulative</b>
Subtotal _____	
Indirect Costs _____	
Fee _____/_____	
Total _____	

10. Comments on administrative and logistical matters. NONE
11. Use additional page(s), as necessary, to describe scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this assistance agreement.
12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.